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Microbubble Augmented Ultrasound Sonothrombolysis Decreases Intracranial Hemorrhage in a Rabbit Model of Acute Ischemic Stroke

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Abstract

Objectives—Increasing evidence confirms microbubble (MB) augmented ultrasound (US) thrombolysis enhances clot lysis with or without tissue plasminogen activator (tPA). Intracranial hemorrhage (ICH) is a major complication militating against tPA use in acute ischemic stroke. We quantified the incidence of ICH associated with tPA thrombolysis and MB+US therapy and compared infarct volumes in a rabbit model of acute ischemic stroke.

Materials and Methods—Rabbits (n=158) received a 1.0-mm clot angiographically injected into the internal carotid artery causing infarcts. Rabbits were randomized to six test groups including: 1) control (n=50), embolized without therapy, 2) US (n=18), 3) tPA only (n=27), 4) tPA +US (n=22), 5) MB+US (n=27), and 6) tPA+MB+US (n=14). Ultrasound groups received pulsed wave US (1 MHz, 0.8 W/cm²) for 1 hour; rabbits with tPA received intravenous tPA (0.9 mg/kg) over 1 hour. Rabbits with MB received intravenous MB (0.16 mg/kg) given over 30 minutes. Rabbits were sacrificed 24 hours later and infarct volume and incidence, location, and severity of ICH were determined by histology and pathological examination.

Results—Percentage of rabbits having ICH outside the infarct area was significantly decreased ($P=0.004$) for MB+US (19%) rabbits compared with tPA+US (73%), US only (56%), tPA (48%), tPA+MB+US (36%), and control (36%) rabbits. Incidence and severity of ICH within the infarct did not differ ($P>0.39$). Infarct volume was significantly greater ($P=0.002$) for rabbits receiving US ($0.97\pm0.17\%$) than for MB+US ($0.20\pm0.14\%$), tPA+US ($0.15\pm0.16\%$), tPA ($0.14\pm0.14\%$), and tPA+MB+US ($0.10\pm0.20\%$) rabbits; these treatments collectively, excluding US only, differed ($P=0.03$) from control ($0.45\pm0.10\%$).

Conclusions—Treatment with MB+US following embolization decreased the incidence of ICH and efficacy was similar to tPA in reducing infarct volume.

Keywords

Hemorrhage; Ischemic stroke; Microbubbles; Thrombolysis; Ultrasound

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INTRODUCTION

Ultrasound (US)-facilitated thrombolysis with microbubbles (MB) enhances tissue plasminogen activator (tPA) thrombolysis in acute ischemic stroke patients.¹⁻³ Use of MB +US without tPA has been demonstrated to thrombolyse clot in vitro⁴ and in animal models.⁵⁻⁶ Additionally, tPA+MB+US improves clinical outcome in acute ischemic stroke.¹

Intracranial hemorrhage (ICH) is associated with tPA administration both with⁷ and without⁸ US. Increased rates of asymptomatic hemorrhagic transformation have been reported in acute ischemic stroke patients receiving combined US, tPA, and MB.⁹ Enhanced recanalization attributed to tPA+MB+US is associated with increased rates of asymptomatic hemorrhagic transformation with no increased risk of symptomatic ICH.¹⁰ Molina and others did not find increased rates of symptomatic ICH attributed to US-facilitated thrombolysis with MB and tPA.¹⁻³ However, increased bleeding in the recently terminated transcranial US in clinical sonothrombolysis trial¹¹ provides the impetus for investigating the incidence, severity, and clinical significance of ICH involving US-facilitated thrombolysis with MB and tPA, especially at high MB dose rates.

Although asymptomatic and symptomatic ICH have been used as end points following tPA +MB+US therapy in pilot studies,^{1-3, 9} there is a paucity of information relating the incidence of ICH to MB+US administration with or without thrombolytics in an animal model of acute stroke. Objectives were to determine the incidence of ICH and to quantify infarct volume following various thrombolytic therapies in an angiographic rabbit model of acute ischemic stroke.

MATERIALS AND METHODS

Animals

Animal procedures were approved by the Institutional Animal Care and Use Committee. Female (n=114) and male (n=44) New Zealand White rabbits (n=158; mean body weight=5.2±0.07 kg) were used.

Surgical Procedures

Surgical and angiographic procedures for this model of small to moderate sized strokes were previously described.¹²⁻¹⁴ Rabbits were sedated with an intramuscular injection of ketamine, 30 mg/kg (Ketaset; Fort Dodge; Fort Dodge, IA) and xylazine, 3 mg/kg (AnaSed; Lloyd Laboratories; Shenandoah, IA) and anesthetized with isoflurane (Novaplus; Hospira Inc.; Lake Forrest, IL). A femoral artery was exposed, and utilizing a 3F vascular sheath, a modified 65-cm angled-tip 3F catheter (SlipCath; Cook Inc.; Bloomington, IN) was placed into the artery. The 3F catheter was advanced over a 0.025-inch angled-tip guide wire (Glidewire; Terumo Medical Corp.; Elkton, MD) under fluoroscopy to the internal carotid artery (ICA).

Sub-selective ICA magnification angiography was performed in lateral and frontal projections to document the cerebral vasculature prior to embolization (Figure 1A). Embolization was accomplished by injection of a clot into the ICA. Flow usually carried the clot to the middle cerebral artery (Figure 1B). One minute following embolization, repeat angiography was performed and the location of occlusion was recorded using a single plane C-arm digital subtraction angiography machine (OEC 9800; GE Healthcare; Salt Lake City, UT).

Immediately following embolization and angiography, treatment was initiated. An intravenous catheter (Instyle-W; Becton Dickinson; Sandy, UT) placed into an ear vein was used for tPA and MB administration. Following the procedure the 3F arterial catheter was removed and the incision was sutured. Rabbits recovered and were monitored for adverse effects.

Preparation of Embolus

Emboli were prepared by obtaining an arterial blood sample (2-mL) from a donor rabbit and immediately transferred into a 30.5-cm length of Butterfly pediatric infusion set (No. 4506; Abbott Hospitals, Inc.; North Chicago, IL) with an internal diameter of 1.0-mm and allowed to clot at 37°C for 3 hours. The clot was expelled from the tubing into a dish containing physiological saline and cut into pieces 1.0-mm in length with an approximate diameter of 0.6-mm. A single clot was drawn into a 3.0-mL syringe containing physiological saline for injection into the ICA with 0.7 to 2.0 mL flush.

Ultrasound and Treatments

Rabbits with US received transcutaneous pulsed-wave (20% duty cycle) US at 1 MHz, 0.8 W/cm² (mechanical index of 0.16) (Sonicator 716; Mettler Electronics; Anaheim, CA) for 1 hour. Prior to embolization the right side of the head was clipped of hair and depilatory cream applied. Following embolization a hand-held 10-cm² therapeutic transducer was placed in front of the ear and behind the eye and was coupled to the skin with US gel. Transducer aim toward the brain was confirmed fluoroscopically. Ultrasound was administered as described below.

Rabbits were randomly assigned to one of six treatment groups: 1) control (n=50), 2) US (n=18), 3) tPA only (n=27), 4) tPA+US (n=22), 5) MB+US (n=27), and 6) tPA+MB+US (n=14). Mean body weights did not differ among groups and ranged from 5.0 to 5.3 kg. Control rabbits were embolized but received no therapy. Rabbits administered tPA (Cathflo Activase; Genentech; South San Francisco, CA), received standard dose intravenous tPA (0.9 mg/kg) with 10% given as bolus and 90% administered over 60 minutes. Rabbits administered MB received intravenous perflutren lipid MB (Definity; Lantheus Medical Imaging; North Billerica, MA) at a dose rate of 0.16 mg/kg, which is higher than standard imaging doses by 10-fold.

Microbubbles were activated using an automated shaker for 45 seconds. After activation, each mL of the suspension contained 1.2×10^{10} perflutren lipid microspheres (diameter range=1.1 to 3.3 μ m), and about 150 μ L/mL (1.1 mg/mL) octafluoropropane. The required dose (0.16 mL/kg body weight) was withdrawn and diluted to 6.0-mL with physiological saline and the mixture was administered over 30 minutes in divided boluses every 5 minutes.

Functional Testing

Twenty-four hours following embolization, rabbits were sacrificed by intravenous administration of 1.5-mL of pentobarbital (Euthasol; Virbac Corp.; Fort Worth, TX). Prior to sacrifice, each rabbit received a neurological assessment score (NAS) using the wryneck test procedure.¹⁵ The NAS is a composite score comprising of motor, sensory, balance, and reflex measures and ranges from 0 to 10, with greater (≥ 5) scores indicating more severe neurological injury.

Measurement of Infarct Volume

Following sacrifice, the brain was harvested, immediately placed in chilled physiological saline for 60 minutes, and then sliced coronally at 0.4-cm intervals using a chilled brain mold (RBM-7000C; ASI Instruments Inc.; Warren, MI). Coronal brain sections (n=8) were

placed in 1% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich; St. Louis, MO) for 45 minutes at 37°C, fixed in 10% formalin, and digitally photographed (Figure 2). Areas of infarction were measured using digital analysis (NIH ImageJ). Each brain section volume was calculated by multiplying the section area by the slice thickness (0.4 cm). Similarly, the infarct volume for each section was calculated by multiplying the area(s) of infarction by the slice thickness. Percent infarct volume was calculated by dividing the sum of the infarct volumes into the sum of the section volumes and multiplying by 100%.

Hemorrhage Determination

Brain sections were fixed in 10% formalin, embedded in paraffin, sectioned at 4- μ m, stained with hematoxylin and eosin (H&E) and evaluated by a veterinary pathologist blinded to the treatments. Intracranial hemorrhage was defined as extravasation of erythrocytes and fluid into the extravascular space. Presence or absence and location of ICH were recorded. Location included ICH within the infarct or outside the infarct area (Figure 3). To assess hemorrhage severity, a semi-quantitative score ranging from 0 (no hemorrhage) to 4 (severe hemorrhage) was assigned to each H&E-stained brain section where a score of 1=extravasation of erythrocytes into Virchow Robin's space, not greater than 50 μ m in diameter and with total area affecting less than 5% of tissue area within a single H&E section (Figure 4A,B), 2=hemorrhage greater than 50 μ m in diameter affecting 5 to 10% of tissue area within a H&E section (Figure 4C,D), 3=hemorrhage affecting 10 to 20% of tissue area within a H&E section (Figure 4E,F), and 4=hemorrhage affecting greater than 20% of tissue area within a H&E section. Each section was scored separately and the final hemorrhage score used for statistical analysis was the average score for sections having hemorrhage.

Statistical Analysis

Data were analyzed by ANOVA utilizing the GLM procedure of SAS (SAS Inst. Inc.; Cary, NC). Least squares means were compared using the PDIF statement (ADJUST=Bonferroni) of SAS when protected by a significant ($P<0.05$) treatment effect. Chi-square analysis, using the FREQ procedure, was used to determine the influence of treatment on incidence of ICH. Pearson correlations were used to evaluate the associations among the variables measured.

RESULTS

Percent infarct volume was influenced ($P=0.002$) by treatment; infarct volume was greater ($P<0.05$) for rabbits treated with US only than for rabbits treated with tPA, tPA+US, MB+US, and tPA+MB+US (Table 1). The other treatments collectively, excluding US only, differed ($P=0.03$) from control. Although the NAS did not differ ($P=0.69$) across treatments (Table 1), the NAS was positively correlated with infarct volume ($r=0.21$; $P=0.0087$).

Percentage of rabbits having ICH in areas of infarct did not differ ($P=0.39$) across treatments and averaged 8% (12/158; Table 2). Hemorrhage severity score within the infarct averaged 1.1 ± 0.3 and did not differ ($P=0.93$) among rabbits having ICH in areas of infarct.

Treatment influenced ($P=0.004$) the incidence of rabbits with evidence of ICH in brain regions outside the infarct; percentage of rabbits with ICH in regions outside the infarct was decreased for MB+US rabbits compared with tPA, tPA+US, tPA+MB+US, US, and control rabbits (Table 2). Hemorrhage was seen both remote and adjacent to the infarct. The pattern did not vary among groups. Hemorrhage severity score in areas outside the infarct did not differ ($P=0.19$) across treatments and averaged 1.1 ± 0.06 (Table 2). Infarct volume was not

correlated ($P>0.729$) with the hemorrhage severity score within the infarct or in areas outside the infarct.

DISCUSSION

Intracranial hemorrhage is the major complication of intravenous tPA administration⁸, and the nature of ICH following ischemic stroke treatment, especially within the penumbra, is not fully understood. Relations to duration of ischemia, other factors, and various therapies need characterization. The major finding of the present study was decreased incidence of ICH outside the infarct, presumably in the penumbra, associated with MB+US treatment.

Daffertshofer et al⁷ reported increased symptomatic hemorrhage with combined transcranial low-frequency (300 kHz; pulsed-wave) US and tPA whereby 5 of 12 (42%) patients from the tPA only group and 13 of 14 (93%) patients from the tPA+US group showed signs of hemorrhage on magnetic resonance imaging (MRI). This included bleeding outside areas of stroke and some deaths that resulted in premature termination of the study. Our study shows the highest incidence of ICH in areas outside the infarct in rabbits treated with tPA+US. Additionally, rabbits treated with tPA+US and tPA alone had a higher incidence of ICH in areas outside the infarct than control rabbits and both were also much higher than for rabbits treated with MB+US. Interestingly, the increased ICH with tPA+US treatment in the study by Daffertshofer et al⁷ and in our study occurred with very different methods of US application. Low-frequency US complications observed by Daffertshofer et al⁷ are possibly a result of focused reflections of long-range 300kHz US waves. At 1-MHz the range in rabbits is shorter minimizing focused reflections, but the greatest incidence of asymptomatic ICH seen in the present study was observed with tPA+US and US alone indicating active impact.

Molina et al¹ reported asymptomatic ICH rates of 16, 19, and 23% in patients receiving tPA, tPA+US, and tPA+MB+US, respectively. Alexandrov et al² reported greater rates of asymptomatic ICH for tPA+MB+US (3/12 [25%]) and tPA+US (1/3 [33%]) treated patients. Asymptomatic hemorrhagic transformation was observed by Viguier et al in 6 of 8 patients treated with tPA+MB+US.⁹ The observations of these investigators are all based on continuous transcranial Doppler 2-MHz US patient monitoring. In the current study, using very sensitive histology instead of the CT imaging used clinically, the percentage of rabbits treated with tPA+MB+US having ICH within the infarct and in regions outside the infarct was 14 and 36%, respectively. Furthermore, a higher percentage of rabbits treated with tPA and tPA+US had histological evidence of ICH in areas outside the infarct than rabbits treated with tPA+MB+US. Only treatment with MB+US resulted in a decreased rate of ICH, a possibly important step toward decreased symptomatic hemorrhage.

The exact mechanisms whereby treatment of rabbits with MB+US results in decreased ICH outside the infarct remain to be delineated. A possible explanation is that the high dose of MB used here is absorbing potential hemorrhage-inducing US energy while more promptly lysing thrombus and restoring blood flow at many levels. Administration of high peak rarefactional pressure US has been demonstrated to lead to nonlinear oscillations of MB, resulting in continuous absorption of US energy, culminating in MB destruction with concomitant release of absorbed energy.¹⁶ Alternatively, MB may be attenuating the US beam and the tissue heating induced by US. Increased temperature of the soft tissue around occluded arteries, substantial damage to rabbit dermis and subcutaneous tissue, and severe histological changes have been reported following application of continuous wave, 20-kHz, 1.5 W/cm² US.¹⁷ Whether MB+US at different dose rates and frequencies than those utilized in the present study results in decreased ICH in animal and human studies remains to be investigated.

A better quantitative assessment of hemorrhage volume and severity is warranted in animal studies of acute ischemic stroke. We evaluated the degree of hemorrhage severity by assigning a semi-quantitative score to each brain section having hemorrhage based on the area of hemorrhage observed microscopically. This is quite different from the clinical system based on CT imaging and cannot be correlated to that at this time. However, this system may aid in comparison of bleeding findings but longer term follow-up, and multiple imaging studies, will be required to prove the importance of these microscopic abnormalities.

Safety studies of MB+US for the treatment of stroke must address hemorrhage; however, animal studies evaluating effects of MB+US on incidence of ICH are limited. Lack of severe hemorrhage in this series of rabbits indicates this type of US maybe safe with all of these applications. However, studies employing extended (>24 hours) animal survival times following embolization are warranted to assess possible worsening of small areas of hemorrhage. A study by Christoforidis et al¹⁸ has recently published a similar minimally invasive embolic model in canines that could be used in future studies.

In human strokes, symptomatic ICH often occurs more than 24 hours after stroke onset. Hemorrhage severity in the present study was much less pronounced than the hemorrhage reported by Daffertshofer et al⁷. However, this model of mild stroke may not have been severe enough to duplicate those human findings in very severe stroke.

Mean infarct volume was greatest for rabbits treated with US alone. Cintas et al¹⁹ reported that relative weight reduction of clot in vitro was less after exposure to US (2 MHz) alone (6%) than tPA+MB+US (31%), tPA+US (13%), and MB+US (11%). Decreased in vitro clot lysis by US alone and decreased revascularization may partially explain why rabbits treated with US alone had a greater infarct volume. Relative weight reduction of clot in vitro was similar between tPA+US and MB+US¹⁹ and this observation may explain why rabbits treated with tPA+US and MB+US had similar infarct volumes. In contrast to the effect of US alone, MB+US decreased infarct volume in the current study. This result is in agreement with observations of Fatar et al²⁰, who reported a decrease in infarct volume following MB+US administration in a rat model of cerebral ischemia. Mechanisms of action of MB+US enhanced thrombolysis are not fully understood but are believed to involve cavitation processes including microstreaming around the MB during stable cavitation and microjetting during unstable cavitation, both of which lead to a destabilized clot structure.²¹⁻²² Additionally, MB can enhance fibrinolysis by mechanically disrupting clots exposed to US, allowing increased penetration of tPA into the fibrin network.²¹ Although not statistically significant, rabbits treated with tPA+MB+US had a numerically lower infarct volume than rabbits treated with MB+US.

As Donnan discussed in the 2007 Feinberg Lecture²³, rodent models have not yet successfully translated into human therapies. However, a rabbit model has led to human tPA therapy.²⁴ We tailored that model to use our angiographic strengths rather than use rodents. Differences in collaterals and a three fold higher glucose and oxygen metabolism, and blood flow rate in rats, compared to humans, may explain why rodents respond more favorably to neuroprotection than higher mammals.²⁵ These differences, coupled with the physical limitations of size, make the rabbit a more attractive model for study.

Study limitations include absence of follow-up beyond 24 hours. To better imitate late-onset ICH in humans and understand the pathophysiological sequelae of the ischemic brain following tPA+MB+US treatment, studies will require the survival of animals past 3 days. Survival protocols will need to include imaging strategies such as MRI to better assess the evolution of brain injury and progression of ICH. To reliably occlude either a single or

duplicated middle cerebral artery, we initially utilized a small clot for embolization. Consequently, the small infarct volumes may prevent adequate separation of results of treatments with practical numbers of animals. Further, clots utilized for embolization were fresh and may have been more amenable to lysis than typical clots in humans. While this model of moderate stroke has value in these roles, older and larger clots may provide a better model of more severe strokes and are now in development.

In conclusion, only treatment with MB+US decreased the incidence of ICH while producing reductions in infarct volumes similar to those obtained with other therapies including standard tPA therapy.

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Figure 1. Cerebral angiogram showing complete filling of a middle cerebral artery (MCA) prior to embolization (A). Subtracted angiogram one minute following embolization shows MCA occlusion (arrow) (B).

Figure 2.
TTC-stained brain section showing infarction (arrow).

Figure 3. Histopathological sections, stained with hematoxylin and eosin (H&E), of the rabbit brain showing hemorrhage within infarct (A) and outside infarct (B).

Figure 4.

Histopathological sections of the rabbit brain showing hemorrhage following H&E staining. A hemorrhage severity score ranging from 0 (no hemorrhage) to 4 (severe hemorrhage) was assigned to each H&E-stained section. A and B are grade 1; C and D grade 2; and E and F grade 3.